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Identification of a thrombospondin-like immunodominant and phosphorylcholine-containing glycoprotein (GP300) in *Dictyocaulus viviparus* and related nematodes

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ABSTRACT

GP300 is a high molecular weight glycoprotein of the bovine lungworm *Dictyocaulus viviparus*. The N-linked glycans are substituted with phosphorylcholine (PC) giving it immunomodulatory potential. GP300 is highly immunogenic and its recognition by IgE antibodies is correlated with protection against infection. Here we identified and characterized the protein backbone of GP300. Mass spectrometric analysis on purified GP300 and DNA sequencing of the corresponding gene indicated that GP300 is a thrombospondin-like protein with 7 thrombospondin domains, 6 kunitz domains and 15 putative N-glycosylation sites. Purified GP300 display protease inhibitory activity. The protein was located in the brushborder of the gut, but also in muscles, hypodermis and the lining of the uterus. Analysis of GP300 orthologues in *Haemonchus contortus* and *Cooperia oncophora* revealed that these proteins also contain PC-substituted N-glycans and showed immunological cross-reactive responses. These data suggest the existence in nematodes of a GP300 protein family that is characterized by PC-substituted N-linked glycans attached to a thrombospondin-like protein backbone. This finding is of particular interest considering the immunomodulatory and vaccine potential of members of the GP300 family.

1. Introduction

Parasitic helminths are well adapted to their host and have evolved smart strategies to modulate the immune system. One class of immunogenic and immune-modulating structures found in nematodes is N-linked glycans. These molecules often show core α(1,3) fucosylation [1,2] or are substituted with phosphorylcholine (PC) [3,4]. The decoration of N-glycans with PC seems exclusive for nematodes and occurs in many, may be even all nematodes. These types of molecules are absent in the host and aid the worms to create their optimal niche [5,6]. To date, the best studied PC containing glycoprotein is ES-62 from the filarial worm *Acantocheilonema viteae*. ES-62 interacts with B and T cells, dendritic cells and macrophages, resulting in an anti-inflammatory response [7,8]. PC is the primary immunomodulating moiety of ES-62; ES-62 without PC display no [9,10], or much less immunomodulatory activity [11], while BSA [9] and OVA [10] are also transformed to immunomodulating proteins by the addition of PC. To our knowledge, ES-62 is also the only glycoprotein with a PC-substituted N-glycan of a parasitic nematode of which the protein backbone has been characterized and cloned. It is an aminopeptidase, produced and excreted in the gut [12,13].

The nematode *Dictyocaulus viviparus* (superfamily Trichostrongyloidea) causes parasitic bronchitis in cattle. The third-stage larvae (L3) of this nematode are taken up orally and the larvae penetrate the intestinal wall. From there they migrate via the mesenteric lymph nodes and the blood circulation to the lungs, penetrate the lung epithelium, and mature to their adult stage in the bronchioles and bronchi. The eggs they produce are coughed up, swallowed, and excreted as first-stage larvae that develop into L3 on the pasture. The clinical symptoms associated with the infection are caused by the larvae, adult worms and/or eggs in the lungs that induce a local influx and activation of eosinophils and mast cells. This may restrict the airways and can result in edema and emphysema and, in severe cases, death of the host.

Infection with *D. viviparus* ultimately generates a protective immune response [14]. Short-lived protection can also be achieved by vaccination with irradiated L3 larvae [15] or by transfer of

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serum from infected animals, indicating that protection is achieved via immunoglobulins [16]. The antibody responses both in primary infected and vaccinated animals are predominantly directed against N-glycans [17]. The immunodominant antigen is GP300 and its recognition by IgE is correlated with protection [18]. This glycoprotein is the only protein in adult *D. viviparus* containing PC-substituted N-linked carbohydrates [19]. Antibodies elicited by primary *D. viviparus* infection or by vaccination are mainly directed against this PC molecule. Furthermore, antibodies of the IgG1 and IgA isotype directed against PC-containing N-linked glycan of GP300 cross-react with and possibly neutralize platelet-activating factor (PAF) which may limit inflammation and aid the bias towards the Th2 immune response [19]. Thus, the PC moiety of GP300 may have immunomodulatory potential.

The nature and possible function of the protein backbone of GP300 to which the PC-substituted N-glycans are attached, are still unknown. Here we present mass spectrometry and cDNA sequence results that indicate that the protein backbone of GP300 is a thrombospondin-like protein. Functionally, the protein displays protease inhibitory activity. In addition, evidence is provided that GP300 orthologues are present in other nematodes, that these glycoproteins are also decorated with PC, and that antibodies directed against the PC moiety and the peptide backbone cross-react between nematodes.

2. Materials and methods

2.1. Parasites

Adult lungworms and L3 larvae of *D. viviparus* were collected from lung washings of infected calves at day 35 of infection as described previously [17,20]. For immunohistochemistry, fresh adult worms were fixed overnight in 4% formaldehyde in phosphate buffered saline (PBS) and then transferred to 70% ethanol. Adult *H. contortus* from abomasum of sheep and adult *C. oncophora* parasites were collected from small intestine of cattle as described [21]. *Toxocara canis* and *Taenia taeniaeformis* adults were obtained from a dog and a cat, respectively, and generously provided by the department of Pathobiology (Faculty of Veterinary Medicine, Utrecht, The Netherlands). Water-insoluble extract from all parasites were prepared as described previously [19].

2.2. Purification of GP300

GP300 was purified from adult worms stored at −80 °C using Wheat Germ Agglutinin (WGA) lectin affinity chromatography as described [19].

2.3. Deglycosylation of glycoproteins

Deglycosylation of glycoproteins kept in solution was achieved by treatment with peptide-N-glycosidase F (PNGase F) as described [19]. In some experiments, extracts were separated on SDS-PAGE and transferred onto nitrocellulose prior to deglycosylation to allow direct comparison of protein bands with and without glycosylation. For deglycosylation of blotted glycoproteins the following adjustments to the blotting and above described deglycosylation procedures were made: (1) Blots were not boiled prior to deglycosylation (2) One blot (8 × 6 cm) was deglycosylated in 20 ml buffer containing 40 μl PNGaseF, (3) Incubation was stopped by extensive washing with Tris-buffered saline containing 0.05% Tween-20 (TBS-T), pH 7.4, and (4) Blots were quenched with TBS-T containing 0.1% gelatine after deglycosylation. A mock treated blot (in which the PNGase F was omitted from the buffer) was always simultaneously incubated to correct for possible loss of proteins during the deglycosylation step.

2.4. Production, affinity purification, and biotinylation of antibodies

The (sheep) anti-rHc-TSP antibody, directed against recombinant thrombospondin of *H. contortus* [22], was generously provided by Dr. P. Skuce from Moredun Research Institute, UK. GP300-specific antibodies were affinity purified from serum of primary infected calves using immobilized GP300 as a matrix, as previously described [19]. Western blots and competitive ELISA demonstrated that the GP300-specific antibodies were directed against PC. The PC-specific monoclonal antibody TEPC-15 (Sigma) served as positive control for detection of PC containing glycoproteins. Anti-GP300 and TEPC-15 antibodies were biotinylated with EZ-link sulfo-*N*-hydroxysuccinimide-biotin (Pierce) according to the instructions of the manufacturer.

2.5. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were carried out as described [17]. Blots were incubated with antibodies or lectins diluted in TBS-T. The following incubation steps were used: (1) Biotinylated-WGA (Pierce, dilution: 1/100,000) followed by streptavidin-HRP (Pierce, dilution: 1/2000), and staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in the presence of nickel [23], (2) anti-GP300 (0.5 μg/ml) followed by mouse anti-bovine IgG1 (mca627, Serotec, dilution: 1/1000), (3) TEPC-15 (Sigma, dilution: 1/1000), or (4) Sheep anti-rHc-TSP (dilution: 1/1000), followed by mouse anti-ovine IgG (mca 893, Serotec, diution: 1/500). Probes 2-4 were detected with alkaline phosphatase conjugated goat anti-mouse Ig (DAKO, dilution 1/2000) using BCIP and NBT (Sigma) as a substrate.

2.6. Mass spectrometry

For mass spectrometric analysis, WGA-purified GP300 was diluted in SDS-PAGE sample buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue R250 (0.1%, w/v) staining solution. The two protein bands were excised from the gel, rinsed with distilled water, and subjected to in-gel tryptic digestion as described previously [24] with one minor modification, namely that gel pieces were submerged in 50 μl of ammoniumbicarbonate buffer during overnight trypsin digestion (37 °C). After digestion, 10 μl of the supernatant was analyzed by nanoflow-LC tandem mass spectrometry using an Agilent 1100 HPLC (Agilent Technologies) coupled to an LTQ ion trap mass spectrometer (Thermo Electron, Bremen, Germany) as described previously [25]. For all protein band analysis *.dta files were created using Bioworks 3.1 software (Thermo Electron, Bremen, Germany) which were converted to a single peak list in Mascot generic format using in-house developed software. For protein identification, an in-house Mascot server [26] (Mascot version 2.0 search software; Matrix Science, London, UK) was used to search the MSDB (version 20050701) database (http://csc-fserve.hh.med.ic.ac.uk/msdb.html), allowing a peptide mass tolerance of 0.6 Da, fragment mass tolerance of 0.6 Da, and allowing for two missed cleavages. Modifications included in the search were carbamidomethyl at cysteine residues (as a fixed modification), oxidation at methionine at residues (variable) and a +1.0 amu mass change at asparagine residues (variable).